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Subdivision of the *Pestivirus* Genus Based on Envelope Glycoprotein E2

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Conventionally, the genus *Pestivirus* of the family *Flaviviridae* has been divided into bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV). To date, BDV and BVDV have been isolated from different species, whereas CSFV seems to be restricted to swine. Pestiviruses are structurally and antigenically closely related. Envelope glycoprotein E2 is the most immunogenic and most variable protein of pestiviruses. We cloned E2 genes of many different pestivirus strains, including those from a deer and a giraffe. The E2 genes were transiently expressed, characterized with monoclonal antibodies, sequenced, and compared. Based on these data, we can delineate six major groups within the *Pestivirus* genus. Four groups correspond to defined genotypes, whereas the two other groups could be new genotypes within the *Pestivirus* genus. One group comprises CSFV strains isolated from swine. A second group consists of BDV strains Moredun, L83, and X818, which have been isolated from sheep, and strain F from swine. A third group contains strain BD78 from sheep, strain 5250 from swine, and strain 178003 from cattle. On the basis of E2, these viruses are very similar to BVDV strains associated with acute severe outbreaks of bovine viral diarrhea, so-called type 2 BVDV. The fourth group consists of BVDV strains originating predominantly from cattle. This BVDV group can be divided into two subtypes or subgroups BVDV Ia and Ib: BVDV Ia contains viruses from the United States, such as like NADL and Oregon, and some others, such as 150022 and 1138 from Europe. Subgroup BVDV Ib contains strain Osloss and several Dutch isolates. The fifth and sixth "groups" could be proposed as two new genotypes and contain strains Deer and Giraffe, respectively.

INTRODUCTION

The genus *Pestivirus* of the family *Flaviviridae* consists of classical swine fever virus (CSFV), border disease virus (BDV), and bovine viral diarrhea virus (BVDV). Genomes of several BVDV and CSFV strains have been sequenced (Renard *et al.,* 1987; Collett *et al.,* 1988; Deng and Brock, 1992; Ishikawa *et al.,* 1995; Meyers *et al.,* 1989; Moormann *et al.,* 1990, 1996). For BDV, only incomplete genomic nucleotide sequences are available (Becher *et al.,* 1994; Sullivan *et al.,* 1994). The pestivirus genome is a positive-stranded RNA molecule of about 12.5 kb containing one large open reading frame. The open reading frame is translated into a hypothetical polyprotein of approximately 4000 amino acids, which is processed by virus- and cell-encoded proteases. The 5′-terminal part of the open reading frame encodes one nonstructural protein and four structural proteins, the nucleocapsid C, and three envelope glycoproteins, E^{RNS}, E1, and E2. The open reading frame is flanked by two highly conserved small nontranslated regions, which are probably involved in the replication of the genome. The 5′-noncoding region also plays a role in initiation of translation (Poole *et al.,* 1995; Rijnbrand *et al.,* 1997).

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After the discovery and characterization of several pestiviruses, the originating host was used as a criterion to subdivide pestivirus strains. However, so far only CSFV appears to be restricted to a single host, whereas other pestiviruses naturally occur in several animal hosts; e.g., BVDV infects cattle, sheep, and swine (Paton *et al.,* 1995). Later, many other methods were explored to subdivide pestiviruses. Comparison of the nucleotide sequences of the 5′-noncoding region has been used to divide pestivirus strains into genetic groups (Harasawa, 1994; Vilcek *et al.,* 1994; Harpin *et al.,* 1995; Hofmann *et al.,* 1994; Paton *et al.,* 1995; Pellerin *et al.,* 1994; Wirz *et al.,* 1993). Antisera raised against pestivirus strains have been used to determine four to six neutralization groups (Dekker *et al.,* 1995; Paton *et al.,* 1995). However, this method may be problematic, since, due to their close antigenic relatedness, infections with one pestivirus induce varying extents of antibodies cross-reacting with other pestiviruses (Moennig and Plagemann, 1992).

Many monoclonal antibodies (MAbs) directed against pestiviruses have been developed, but relatively few strains have been used to raise these MAbs (Peters *et al.,* 1986; Wensvoort *et al.,* 1986, 1989b; Moennig *et al.,* 1987; Bolin *et al.,* 1988; Caij *et al.,* 1993; Paton *et al.,* 1991, 1994). One group of MAbs binds to highly conserved pestivirus epitopes on nonstructural protein NS3. A sec-

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ond group of MAbs is directed against conserved CSFV-specific epitopes on structural protein E2 (Wensvoort *et al.,* 1989a). In addition, several CSFV-specific MAbs recognize nonconserved epitopes. Many MAbs have been raised against BVDV and BDV, but none of the recognized epitopes on the structural proteins are absolutely conserved within a particular group of pestiviruses (Paton *et al.,* 1995).

Most MAbs raised against pestiviruses are directed against envelope glycoprotein E2 (Wensvoort, 1989a,b; Weiland *et al.,* 1990; Edwards *et al.,* 1991; Weiland *et al.,* 1992). Epitopes on E2 of CSFV ($E2^{CSFV}$) have been divided into four antigenic domains, domains A–D. Domain A contains epitopes conserved among CSFV, whereas epitopes of the other domains are not completely conserved (Wensvoort, 1989a). All domains are located on the N-terminal half of E2, which consists of two independently formed structural antigenic units (van Rijn *et al.,* 1993, 1994). The immune response induced by $E2^{CSFV}$ is protective against CSFV (van Zijl *et al.,* 1991; Hulst *et al.,* 1993; König *et al.,* 1995). Even more, immunization with deletion $E2^{CSFV}$ proteins containing one structural antigenic unit protects pigs against CSFV (van Rijn *et al.,* 1996). E2 of other pestiviruses has been less extensively studied, but several similarities between $E2^{CSFV}$ and E2 of BVDV ($E2^{BVDV}$) suggest a similar overall structure. Cysteine residues and amino acid mutations observed in escape variants are located on similar positions in $E2^{CSFV}$ and $E2^{BVDV}$ (Paton *et al.,* 1992; van Rijn *et al.,* 1994). Nonetheless, E2 is the most variable gene of pestiviruses. In this paper, E2 genes of pestivirus strains from different hosts and of different neutralization groups were investigated, to subdivide the genus *Pestivirus.*

MATERIALS AND METHODS

Pestivirus strains

Pestivirus strains 150022, 154985, and 178003 and those from deer and giraffe (Plowright, 1969) were provided by Dr. Paton (Paton *et al.,* 1995). BDV strain Moredun was kindly provided by Dr. Nettleton (Edinburgh, Scotland). Other pestivirus strains were from the collection of ID-DLO in Lelystad. Strain 4800 was isolated from a persistently infected calf in 1993 (Bruschke *et al.,* 1996). Strain F was isolated from a pig in 1994. BVDV and BDV strains were propagated in subconfluent monolayers of embryonic bovine tracheal cells, which were incubated for 24 to 96 hr. CSFV strains were propagated in swine kidney cells (SK6-M) (Terpstra, 1989). Calf serum was routinely collected from specified pathogen-free animals and was checked for BVDV and BVDV antibodies.

Amplification and cloning of E2 genes in a suitable expression vector

Total cytoplasmic RNA of infected cells was isolated as described by Chomczynski and Sacchi (1987). To amplify the E2 gene, RNA was reverse transcribed into cDNA by reverse transcriptase and random hexamers according to the supplier (Promega) and amplified [polymerase chain reaction (PCR)] according to standard methods (39 cycles, 94° 1 min, 55° 1 min, 72° 1 min) using a set of primers listed in Table 1 and Taq polymerase (Boehringer-Mannheim) or Vent polymerase (Biolabs).

Amplified E2 fragments were digested with *Spe*I and *Afl*II and ligated into a derivative of pPRc34 (van Rijn *et al.,* 1992). The resulting plasmids were designated pPRKbvd10 to pPRKbvd96 (Table 1). By the presence of internal *Spe*I or *Afl*II sites, the amplified fragment was partially digested with the respective enzyme to clone the entire E2 fragment. The 5′ half of the E2 gene of strain 5250 and of CSFV strains 331 and 371 was amplified by a set of primers overlapping the *Spe*I and the *Bgl*II sites. The latter site is located in the center of the E2 gene of CSFV strain Brescia (Moormann *et al.,* 1990). These fragments were cloned by replacement of the *Spe*I–*Bgl*II fragment of pPEh2 (van Rijn *et al.,* 1992), resulting in plasmids designated pPRKbvd77, pPRvp3, and pPRvp2, respectively. All cloning procedures were carried out essentially as described (Sambrook *et al.,* 1989). Restriction enzymes and DNA modifying enzymes were used as described by the suppliers (Promega and Biolabs). Plasmids were transformed and maintained in *Escherichia coli* strain DH5α (Hanahan, 1985). Plasmid DNA was isolated from overnight cultures and purified by columns (QIA-gen).

Analysis of E2 sequences

The nucleotide sequences of E2 genes were determined by double-stranded DNA sequencing (T7 kit of Pharmacia LKB) or by thermocycle sequencing (Applied Biosystems). Sequencing was performed on at least two independently amplified and cloned fragments. Both nucleotide and deduced amino acid sequences were compared with the PC-GENE program CLUSTAL, GCG PileUp program, MacMolly multialignment program, and multicomparison program of Lasergene. Positions shown are with respect to the nucleotide and amino acid sequences of CSFV strain C (Moormann *et al.,* 1996, EMBL/GenBank database Accession No. Z46258). The nucleotide sequence data for the E2 genes of the different pestivirus strains have been deposited in the EMBL/GenBank database under the following accession numbers: BD112 (Y12174), 150022 (Y12177), 178003 (Y12164), 4800 (Y12167), cpAppel (Y12176), ncpAppel (Y12175), 1138 (Y12166), Korevaar (Y12165), 5250 (Y12168), strain F (Y12169), Deer (Y12170), Giraffe (Y12171), 331 (Y12172), 371 (Y12173).

Transfection and immunoperoxidase monolayer assay

Transfection of COS1 cells (ATCC) was carried out as described (van Rijn *et al.,* 1994). After incubation at 37°

TABLE 1

Primers Used to Amplify E2 Genes of Different Pestiviruses

A

Strain	Set of primers	Plasmid
NADL-L	738–739	pPRKbvd10
BD112	738–739	pPRKbvd11
ncAppel	810–829	pPRKbvd12
Osloss-L	738–739	pPRKbvd21
cpAppel	738–739	pPRKbvd38
Oregon-L	811–813	pPRKbvd40
150022	810–813	pPRKbvd42
178003	810–830	pPRKbvd43
Korevaar	810–828	pPRKbvd44
1138	810–739	pPRKbvd59
4800	810–739	pPRKbvd65
F	810–828	pPRKbvd80
Deer	810–830	pPRKbvd85
Giraffe	810–830	pPRKbvd96

B

Forward primers

738	5′-Tr.TGG.CTG.CTA.CTA.GTA.ACn.GGG.GCA.CAA.GG-3′
810	5′- TGG.CTA.CTA.CTA.GTA.ACA.GGG.GTA.CAA.GG-3′
811	5′- TGG.CTA.CTA.CTA.GTA.ACA.GGA.GTG.CAG.GG-3′

Reverse primers

739	3′-TG.ATA.CTC.nCC.CTT.AAG.CAT.rTA.TTG.-5′
813	3′-TG.ATA.CTC.nCC.CTT.AAG.CAT.GTA.TTG.CTG.-5′
828	3′- TA.CTC.nCC.CTT.AAG.CAT.GTA TTG.yTG.GAA.ATA.-5′
829	3′- TA.CTC.nCC.CTT.AAG.CAT.GTA TTG.yTG.AAA.ATA.-5′
830	3′- TA.CTC.nCC.CTT.AAG.CAT.GTA TTG.yTG.GAA.GTA.-5′

Note. (A) Sets of primers successful in amplification and cloning of E2 genes are shown. Expression plasmids containing *Spe*I–*Afl*II E2 fragments of pestivirus strains are indicated. (B) Nucleotide sequences of forward and reverse primers. G or A, C or T, and G, A, T, or C are indicated by r, y, and n, respectively. The deduced open reading frame is indicated. *Spe*I and *Afl*II sites are underlined.

for 24 hr, transfected monolayers were washed three times with PBS, dried for 45 min at 37°, and frozen at −20° for 45 min. Frozen cells were fixed 5 min with 4% paraformaldehyde in PBS and washed three times with 0.15% NaCl. Immunostaining of transfected cells [immunoperoxidase monolayer assay (IPMA)] was performed essentially as described (Wensvoort *et al.,* 1986). Binding of nonconjugated MAb was visualized by binding of conjugated rabbit anti-mouse antibodies. If no immunostaining was observed, a second staining with swine hyperimmune serum (HIS) or with other MAbs and conjugated rabbit anti-mouse antibodies was performed to check the transfection. HIS was raised in a pig and conjugated with horseradish peroxidase according to standard procedures. The pig was first immunized with CSFV vaccine strain C, followed by infection with BVDV I strain Boomzaaier (Dekker *et al.,* 1995). MAbs WB162, WB166, WB214, WB215, and WB537 were provided by Dr. Paton and Dr. Edwards, and MAbs CA1, CA34, and CT6 by Dr. Moennig and Dr. Bolin. MAbs CA1, WB162, and WB215 were raised against strain Oregon C24V, MAbs WB166 and WB214 against strain NADL, MAb WB537 against strain Vosges, MAb CA34 against strain 7443, and MAb CT6 against strain 1138 (Edwards *et al.,* 1988; Bolin *et al.,* 1988).

RESULTS

Cells infected with the selected virus strains were immunostained with pestivirus-specific HIS, which confirmed that these virus strains belong to the *Pestivirus* genus (Table 2). CSFV-specific MAbs immunostained cells infected with CSFV strains, whereas cells infected with the other pestiviruses were not detected with these MAbs. The reaction pattern with a small panel of MAbs specific for E2 of pestiviruses was unique for each pestivirus, demonstrating the antigenic variability of E2 of pestiviruses (Table 2). None of the epitopes appeared conserved in all strains. For example, the epitope recognized by MAb 166 is highly conserved, but was not detectable on E2 protein of virus strains 178003, 1138, and Deer. With the exception of strain NADL, MAb 162 recog-

TABLE 2

Results of IPMAs of Monolayers Infected with Pestiviruses Using a Small Panel of Monoclonal Antibodies

Virus	HIS	162	166	214	215	CT6	CA1	CA34
NADL-L	+	−	+	+	+	+	−	−
Osloss-L	+	+	+	−	+	−	−	+
Oregon-L	+	+	+	+	+	+	+	+
150022	+	+	+	+	+	−	+	+
154985	+	+	+	−	+	+	+	−
178003	+	−	−	−	−	−	−	−
Moredun	+	−	±	+	−	−	−	−
cpAppel	+	−	+	−	−	−	−	−
ncpAppel	+	−	+	+	−	−	+	+
Korevaar	+	−	+	−	±	−	−	±
W96	+	−	+	+	−	−	+	+
Zijlmans	+	−	+	+	+	+	+	+
4800	+	−	+	+	−	−	+	±
1138	+	+	−	−	+	+	−	−
BD112	+	−	+	−	+	±	−	±
5250	+	−	+	−	−	−	−	−
Wisman	+	−	+	−	−	−	−	+
F	+	−	+	−	−	−	−	±
Deer	+	−	−	−	−	+	−	−
Giraffe	+	−	+	−	−	−	−	−
C	+	−	−	−	−	−	−	−
Brescia	+	−	−	−	−	−	−	−

Note. Monolayers of EBTr cells infected with ruminant pestivirus strains or monolayers of SK6 cells infected with CSFV strains were immunostained with conjugated hyperimmune BVDV antiserum (HIS) or indicated MAbs and conjugated rabbit anti-mouse antibodies. +, positive; ±, weak positive; −, negative.

nized strains from the United States and strain 1138 from Europe. In general, the MAbs bound to several virus strains, but none of them recognized all members of a defined neutralization group (Dekker *et al.,* 1995; Paton *et al.,* 1995). Strain 178003 was recognized only by MAb 537, strain cpAppel only by MAb 166, and Deer only by MAb CT6. In contrast, cells infected with strain Oregon were immunostained by all MAbs used (Table 2).

Pestiviruses from different species and of different neutralization groups were selected to study the variability of the *Pestivirus* genus. The E2 genes of these selected pestivirus strains were amplified, cloned, and transiently expressed in COS1 cells. Special attention was given to generate primers to amplify (PCR) these E2 genes. We compared published E2 sequences and found that the signal sequence and a stretch of amino acid residues located upstream of the transmembrane region (TMR) are highly conserved. Despite the high conservation of amino acids, it was not possible to generate "universal" primers for the amplification of all E2 genes. Nevertheless, with a limited set of primers we amplified E2 genes of many antigenically different pestiviruses (Table 1). All forward primers overlap and contain the *Spe*I site (positions 2419–2424) located in the region encoding the signal sequence of E2 of CSFV strain C. All reverse primers overlap and contain the *Afl*II site located on positions 3395–3400 of strain C. If E2 genes were not amplified with a set of primers listed in Table 1, the use of other primers was investigated. With a reverse primer, overlapping a part of the 3′ half of E2, the 5′ halves of the E2 genes of strains 5250, 331, and 371 were amplified.

Amplified and *Spe*I–*Afl*II-digested cDNA fragments were ligated into an expression plasmid under control of the hCMV promoter. Resulting plasmids contain E2 genes of different pestivirus strains with a hybrid signal sequence and the TMR of strain C. Monolayers of COS1 cells transfected with these plasmids were immunostained with different MAbs, indicating that the hybrid signal sequence and TMR are functional in the expression and detection of E2 proteins (Table 3). Cloning of the cDNA fragments containing the 5′-terminal half of the E2 gene of strains 5250, 331, and 371 resulted in hybrid E2 genes containing the N-terminal half of E2 of the respective strain and the C-terminal half of $E2^{CSFV}$. These hybrid E2 proteins were also detected by immunostaining, which indicates that hybrid E2 genes are expressed and recognized by MAbs (pPRKbvd77, pPRvp2, and pPRvp3) (Table 3).

Immunostaining results with transiently expressed E2 proteins matched those of virally encoded E2 proteins with two exceptions, thereby confirming the origin of the cloned E2 genes (Tables 2, 3). Furthermore, these results confirmed the correctness of the amplification by reverse transcription PCR. The latter was also checked by sequencing of two independently obtained cDNA fragments (see below). In the case of the two exceptions, immunostaining with one MAb was negative for transiently expressed E2, whereas immunostaining of infected cells with the same MAb was positive. Viral $E2^{NADL\text{-}L}$ was immunostained by MAb 214, whereas transiently expressed $E2^{NADL\text{-}L}$ (pPRKbvd10) was not recognized by this MAb. Immunostaining of transiently expressed $E2^{1138}$ (pPRKbvd59) with MAb 162 was negative, whereas this MAb bound to viral $E2^{1138}$. The transiently expressed hybrid E2 protein containing the N-terminal half of $E2^{5250}$ and the C-terminal half of $E2^{CSFV}$ was detected by MAb 166, which indicates that the epitope of MAb 166 is located on the N-terminal half of the $E2^{5250}$ protein. Cells expressing hybrid E2 proteins, which contain the antigenic N-terminal half of E2 of strain 331 or 371, were immunostained by CSFV-specific MAbs. This indicates that strains 331 and 371 are CSFV strains.

A minimum of two independently obtained fragments of unpublished E2 genes were sequenced to check errors introduced by PCR. If differences were observed between two clones, the consensus sequence was determined by sequencing a third independently obtained cDNA fragment. In the case of published sequences, the determined sequence was compared with the published

TABLE 3

Results of IPMAs of COS1 Monolayers Transfected with Expression Plasmids Harboring E2 Genes of Different Pestivirus Strains Using a Small Panel of Monoclonal Antibodies

Plasmid	HIS	162	166	214	215	CT6	CA1	CA34	Originating virus
pPRKbvd11	+	−	+	−	±	±	−	+	BD112
pPRKbvd65	+	−	+	+	−	−	+	±	4800
pPRKbvd21	+	+	+	−	+	−	−	+	Osloss-L
pPRKbvd44	+	−	+	−	+	−	−	+	Korevaar
pPRKbvd12	+	−	+	−	−	−	−	−	cpAppel
pPRKbvd38	+	−	+	+	−	−	+	+	ncAppel
pPRKbvd40	+	+	+	+	+	+	+	+	Oregon-L
pPRKbvd59	−	−[a]	−	−	+	+	−	−	1138
pPRKbvd42	+	+	+	+	+	−	+	+	150022
pPRKbvd10	+	−	+	−[a]	+	+	−	−	NADL-L
pPRKbvd43[b]	−	−	−	−	−	−	−	−	178003
pPRKbvd77[c]	−	−	+	−	−	−	−	−	5250
pPRKbvd80	nd	−	+	−	−	−	−	+	F
pPRKbvd85	nd	−	−	−	−	+	−	−	Deer
pPRKbvd96	nd	−	+	−	−	−	−	−	Giraffe
pPRc34[d]	nd	−	−	−	−	−	−	−	C
pPRb2[d]	nd	−	−	−	−	−	−	−	Brescia
pPRvp2[d]	nd	−	−	−	−	−	−	−	371
pPRvp3[d]	nd	−	−	−	−	−	−	−	331

Note. Immunostaining was carried out with hyperimmune BVDV antiserum (HIS) or indicated MAbs and conjugated rabbit anti-mouse antibodies. +, positive; ±, weak positive; −, negative; nd, not determined.

[a] Difference of detection by MAb between transiently expressed (negative staining) and viral E2 protein (positive staining, see Table 2). Originating virus strains are indicated in the last column. Pestivirus strains are divided into groups as in Fig. 2.

[b] Expression of $E2^{178003}$ was confirmed by positive immunostaining with MAb 537. All other E2 proteins and strains were not recognized by MAb 537.

[c] pPRKbvd77 harbors a hybrid E2 gene of the N-terminal half of strain 5250 and the C-terminal half of CSFV strain C.

[d] Expression plasmids harboring E2 genes of CSFV strains Brescia and C have been described (van Rijn *et al.,* 1992). Plasmids pPRvp2 and pPRvp3 contain the N-terminal half of CSFV strains 371 and 331, respectively. Expression of $E2^{CSFV}$ proteins was confirmed by positive immunostaining with CSFV specific MAbs.

sequence. The E2 gene of strain Osloss-L is missing three thymidine residues in a region of 10 nucleotides, resulting in a deletion of one leucine between amino acid positions 827 and 828 and three amino acid changes on positions 829–831 compared with the published sequence (Renard *et al.,* 1987). The E2 gene of strain Oregon contains one point insertion and one point deletion, resulting in a frameshift and consequently amino acid changes from 823 to 828.

To study the antigenic variation of pestiviruses based on E2, we compared the deduced amino acid sequences. Since the N-terminal part is the most immunogenic part of the E2 protein (van Rijn *et al.,* 1993), we focused on the deduced amino acid sequences corresponding to position 690 (the first amino acid of mature E2) to position 865 (Fig. 1). Compared with CSFV, the E2 protein of other pestiviruses contains two extra cysteine residues at positions 748 and 794 (Fig. 1). In addition to the conserved cysteines, many other amino acid residues are conserved among pestiviruses, and even highly conserved stretches of amino acids were found (Fig. 1). Besides, several insertions or deletions of amino acids were found, which are all located in less conserved regions of E2. In more detail, a deletion of two amino acids between positions 721 and 722 was observed in group BVDV II (Fig. 1, see also Fig. 2). In the amino acid sequences of groups BVDV I, BVDV II, Deer and Giraffe an insertion of one amino acid (valine or isoleucine) between positions 781 and 782 was found with respect to the CSFV and BDV groups.

The antigenic relationship between pestiviruses was studied by comparison of the amino acid sequences of published E2 proteins and E2 proteins determined in this study, since E2 is immunodominant and the most variable protein of pestiviruses. Amino acid sequences of entire E2 and N-terminal halves of E2 proteins were compared. Phylogenetic trees on the basis of these comparisons were similar. On the basis of the amino acid sequences of the N-terminal half of E2, the pestivirus genus could be divided into seven groups (Fig. 2). Six of these antigenic groups correspond to the neutralization groups found by cross-neutralization experiments (Dekker *et al.,* 1995). The homology between the amino acid sequences within these six antigenic groups is mostly more than 80%, whereas the homology between two different antigenic groups is less than 60%. The seventh group pro-

a

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                        *                                                   *           *
NADL            HLDCKPEFSY AIAKDERIGQ LGAEGLTTTW KEYSPG-MKL EDTMVIAWCE DGKLMYLQRC
NADL-L          HLDCKPEFSY AIAKDERIGQ LGAEGLTTTW KKYSPG-MKL EDTMVIAWCE DGKFMYLQRC
NY1             DLHCKPEFSY AIARDERIGQ LGAEGLTTTW KDYSPE-MKL EDTMVIAWCK DGKFTYLPRC
Singer          DLHCKPEFSY AIARDERIGQ LGAEGLTTTW KDYSPE-MKL EDTMVIAWCK DGKFTYLPRC
150022          DLDCKPEYSY AIAKDERIGA LGAEGLTTTW KDYSPE-MML EDTMVIAWCK DGKFTYLPRC
Oregon          HLDCKPEYSY AIAKSDRIGL QGAEDLTTVW KDYSHG-MTL EDTMVIAWCK DGKLTYYARC
Oregon-L        HLDCKPEYSY AIAKSDRIGL QGAEDLTTVW KDYSHG-MTL EDTMVIAWCK DGKLTYYARC
1138            HLDCKPEYSY AIAKSDRIGL QGAEDLTTVW KDYSHG-MTL EDTMVIAWCK DGKLTYYARC
SD1             HLDCKPEYSY AIAKNDRVGP LGAEGLTTVW KDYSHE-MKL EDTMVIAWCK GGKFTYLSRC
R2727           HLDCKPEYLY AIAKSDRIGP LGAEGLTTVW KDYSRE-MVL EDTMVIAWCK DGKFTYLPRC

BD112           YPDCKPDFLY AIAKINEIGP LGATGLTTQW YEYSEG-MRL QDTVVVVWCR DGEFKHLTTC
4800            YPDCKPDFSY AIAKNDEMGP LGATGLTTQW YEFSVG-MRL QDTDVVVWCK DGEFRHLSTC
cpAppel         YPDCKPGFSY AIARNEDIGP LGATGLTTQW YEYSDG-VQL DDTAVAVWCK DGEFRFLTKC
ncpAppel        YPDCKPGFSY AIARNEDIGP LGATGLTTQW YEYSDG-VQL DDTAVAVWCK DGEFRFLTKC
Korevaar        YPDCKPDFSY AIAKNEEIGP LGATGLTTQW YVYSDG-MRL EDTVVVVWCK DGEFRYRIIC
Osloss          LPVCKPGFYY AIAKNNEIGP LGATGLTTQW YEYSDG-MRL QDTGVVVWCK GGEIKYLITC
Olsoss-L        LPVCKPGFYY AIAKNNEIGP LGATGLTTQW YEYSDG-MRL QDTGVVVWCK GGEIKYLITC

Deer            YPKCKPDYSY AIAKNDKVGL LGAEGLTTLW REYKPK-MIL QDALVKVRCK DGEFVHRKKC

178003          FPECKEGFQY AISKDRKMGL SGAGSLTTTW H--PPT-KKI VDSMVQVWCE EKDLKILKTC
BD78            FPECKEGFQY AISKDRKMGL LGPESLTTTW H--LPT-KKI VDSMVQVWCE GKDLKILKTC
5250            FPECKEGFQY AISKATKIGL LGPESLTTTW H--LPT-KKL GGSMVQVWCE GKDLKILKTC

F               QIGCREDFRY ALAKTKEIGL LGAEGLTTTW TDYQRT-FYL DDGTVRATCE RGFFWFREHC
L83             QFACREGYKY ALAKTKEIGP LGAESLTTTW TDYQRGKLEL DDGAVRATCS RGFFKFREHC
Moredun         QFACQEGYRY ALAKTNEIGP LGAESLTTTW TDYQRNNLKL DDGTVRATCS RGFFRFREHC
X818            QFACREDYRY ALARTKEIGA LGAESLTTTW TDY-RGNLEL DDGTVRATCS RGFFRFRGHC

Giraffe         AITCEPEYQY ALARSKRIGP LGAEDLVTTW HDY-KFDLKI QDPLVMVYCK NDQFFVGKRC

Alfort-tub.     RLACKEDYRY AISSTNEIGL LGAEGLTTTW KEYSHGLQ-L DDGTVKAVCT AGSFKVTALN'
371             RLSCKEDYRY AISSTNEIGP LGAEGLTTTW KEYNHGLQ-L DDGTVRATCT AGSFKVIALN
Brescia         RLACKEDHRY AISTTNEIGL HGAEGLTTTW KEYNHNLQ-L DDGTVKAICM AGSFKVTALN
331             RLACKEDFRY AISSTNEIGL LGAEGLTTTW EDYDHNLQ-L DDGTVKAICT AGSFKVTALN
C               RLACKEDYRY AISSTDEIGL LGAGGLTTTW KEYNHDLQ-L NDGTVKASCV AGSFKVTALN
Alfort-187      XLACKEDYRY AISSTNEIGL LGAGGLTTTW KEYNHDLQ-L NDGTVKAICV AGSFKVTALN
WBCSFV          RLACKEDYRY AISSTNEIGL LGAGGLTTTW KEYSHDLQ-L NDGTVKAICV AGSFKVTALN
                .           .           .           .           .           .
                690         700         710         720         730         740

                                                                    * *
NADL            TR-ETRYLAI LHTRALPTSV VFKKLFDGRK QEDVVEMNDN FEFGLCPCDA KPIVRGKFNT
NADL-L          TR-ETRYLAI LHTRALPTSV VFKKLFDGRK QENVVEMNDN FEFGLCPCDA KPIVRGKFNT
NY1             TR-ETRYLAI LHTRALPTSV VFKKLFDGRN EEDVVEMDDN FEFGLCPCDA KPIVRGKFNT
SINGER          TR-ETRYLAI LHTRALPTSV VFKKLFDGRK QEDVVEMDDN FEFGLCPCDA KPIVRGKFNT
150022          TR-ETRYLAI LHTRALPTSV EFKRLFDGQR QKDEVEMDDN FEFGLCPCDA RPVVRGKFNT
Oregon          TR-ETRYLAI LHSRALPTSV VFKKLFEGQG QDYTVEMDDN FEFGLCPCDA KPIVRGTYNT
Oregon-L        TR-ETRYLAI LHSRALPTSV VFKKLFEGQG QEDTVEMDDN FEFGLCPCDA KPIVRGTYNT
1138            TR-ETRYLAI LHSGALPTSV VFKKFFEGQG QEDTVEMDDN FEFGLCPCDA KPIVRGIYNT
SD1             TR-ETRYLAI LHSRALPTSV VFKKLFEGQK QEDTVEMDDD FEFGLCPCDA KPIVRGKFNT
R2727           TR-ETRYLAS LHSRALPTSV KFEKLFEGLR QEDTVEMDDN FEFGLCPCDA KPIVRGKYNT

BD112           ES-EARYLAV LHTRALPTSA VFKRIIDGKT QEDIVDMADN FEFGLCPCDA KPLVRGNFNT
4800            ES-VARYLAV LHTRGLPTSA AFKRIIDGNI QEDIVDMADN FEFGLCPCDA KTVVKGKFNT
cpAppel         ER-EARYLAI LHTKALPTSV VFKKILNGKG QEDIVEMDDN FDFGLCPCDA RPLIRGKFNT
ncpAppel        ER-EARYLAI LHTKALPTSV VFKKILNGKG QEDIVEMDDN FDFGLCPCDA RPLIRGKFNT
Korevaar        ER-EARYLAI LHTRALPTSV VFKKIVNGK- QEDIVEMDDN FEFGLCPCDA KPLVRGKFNV
Osloss          ER-EARYLAI LHTRALPTSV VFEKIIDGKE QEDVVEMDDN FELGLCPCDA KPLVRGKFNT
Osloss-L        ER-EARYLAI LHTRALPTSV VFEKIIDGKE QEDVVEMDDN FELGLCPCDA KPLVRGKFNT

Deer            GK-EARYLAI LHTGALPTSV EFKKLFDKQK QEDIIEMENN FEFGLCPCDA KPWVKERFNN

178003          TK-EERYLVA VHERALSTSA EFMQISEGKI GPEVIDMPDD FELGLCPCDS KPVIRGKFNA
BD78            TK-EERYLVA VHERALSTSA EFMQISDGAI GPDVIDMTDD FEFGLCPCDS KPVIKGKFNA
5250            TR-EERYLVA VHERALSTSA EFMQISDGKL GPSVIDMPDD FEFGLCPCDS KPVIKGKFNA

F               MKGT-RYLAS LHQRALPTSV TFERIPGGST LTEE-EMGDD FEFGLCPCDS RPVVKGKYNT
L83             LIGQ-RYLAS LHQRALPTSV TFELIPGGST LMEE-EMGDD FEFGLCPCDS RPVVKGKYNT
Moredun         LIGQ-RYLAS LHQRALPTSV TFELIPGGST LIKE-EMGDD FEFGLCPCDS RPVVKGKYNT
X818            MIGP-RYLAS LHLRALPTSV TFELIPGGSA MTEE-EMGDD FEFGLCPCDS RPVVKGKYNT

Giraffe         KAGEARYLAK IHWRALPTSV VFEKVLEENP PEEL-PLEDN FDFGLCPCDS RPVVKGKFNT

Alfort-tub      VVSR-RYLAS LHKRALPTSV TFELLFDGTN PAIE-EMDDD FGFGLCPFDT SPVMKGKYNT
371             VVSR-RYLAS LHKRALPTSV TFELLFDGTS PAIE-EMGDD FGFGLCPFDT IPVVKGKYNT
Brescia         VVSR-RYLAS LHKDALPTSV TFELLFDGTS PLTE-EMGDD FGFGLCPYDT SPVVKGKYNT
331             VVSR-RYLAS LHKRALTTSV TFELLFDGTS PSTE-EMGDD FGFGLCPFDT SPVVKGKYNT
C               VVSR-RYLAS LHKKALPTSV TFELLFDGTN PSTE-EMGDD FRSGLCPFDT SPVVKGKYNT
Alfort-187      VVSR-RYLAS LHKEALPTSV TFELLFDGTN PSTE-EMGDD FGFGLCPFDT SPVVKGKYNT
WBCSFV          VVSR-RYLAS LHKGGLLTSV TFDLLFDGTN PSTE-EMGDD FGFGLCPFDT SPVVKGKYNT
                .           .           .           .           .           .
                750         760         770         780         790         800
```

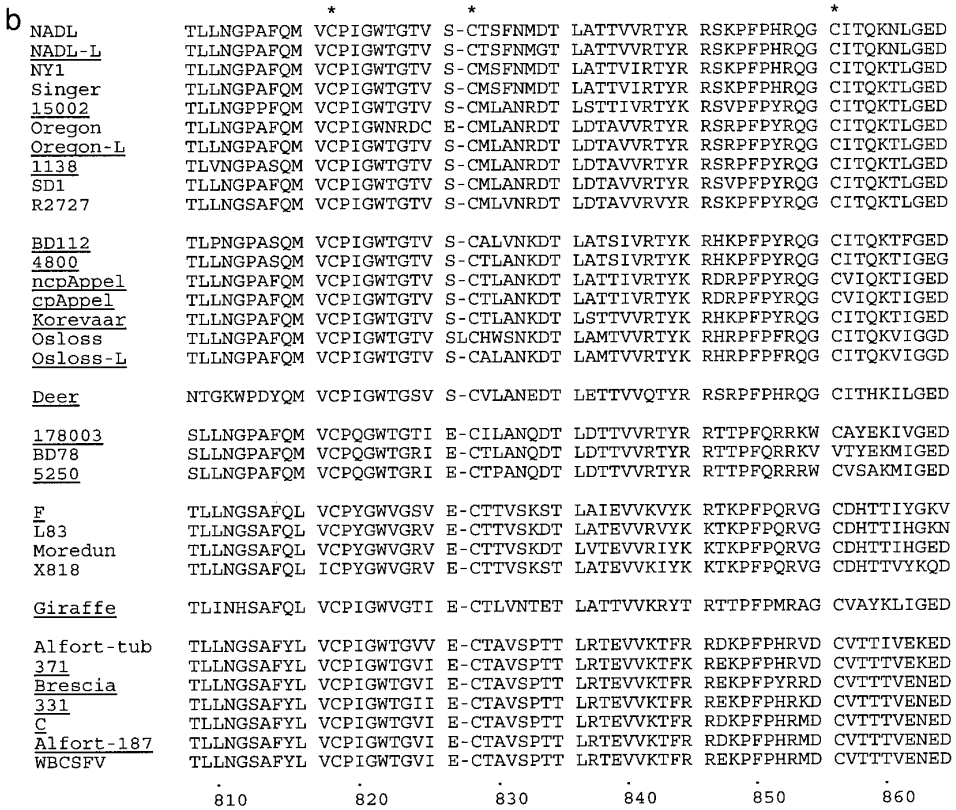

FIG. 1. Comparison of the deduced amino acid sequences of E2 proteins from positions 690 to 865 of the indicated pestiviruses. Pestiviruses are divided into groups as in Fig. 2. Sequences of underlined pestivirus strains were determined in this study. The positions of amino acid residues are based on those of CSFV strain C (Moormann *et al.,* 1996). Cysteine residues are indicated by an asterisk above the alignment.

ceeded from subdivision of antigenic group BVDV I into subgroups BVDV Ia and BVDV Ib. The homology between these subgroups is about 70%, which is intermediate between the homology of two different antigenic groups and that of E2 sequences of viruses belonging to the same antigenic group. One antigenic group consists of CSFV strains, including Alfort-187, Alfort-tub (Meyers *et al.,* 1989), Brescia (Moormann *et al.,* 1990), C (Moormann *et al.,* 1996), 331, the recently isolated Dutch CSFV strain 371, and a CSFV strain from the United Kingdom (WBCSFV). These strains originate exclusively from pigs. A second antigenic group consists of BDV strains Moredun (Barlow, 1972) and L83 and X818 (Becher *et al.,* 1994), all isolated from sheep. This BDV group also contains strain F, which was recently isolated from swine in The Netherlands. A third antigenic group, group BVDV II, consists of strain BD78 from sheep (Sullivan *et al.,* 1994), strain 5250 isolated from swine in 1976, and strain 178003 isolated from cattle in 1987 (Paton *et al.,* 1995). Strain BD78 is very similar to BVDV type 2 strains (Sullivan *et al.,* 1997; Tijssen *et al.,* 1996), indicating that this antigenic group also contains isolates associated with severe outbreaks and high mortalities in veal calves (Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). The fourth antigenic group, group BVDV I, consists of BVDV strains originating predominantly from cattle. This antigenic group can be divided into two subgroups, BVDV Ia and BVDV Ib. Subgroup BVDV Ia contains BVDV strains from the United States, such as NADL and Oregon, and some others, such as 150022 and 1138 from Europe. The second subgroup, BVDV Ib, contains European isolates such as Osloss and several Dutch BVDV isolates from cattle and sheep, such as 4800, Korevaar, Appel, and BD112. Fifth and sixth antigenic "groups" contain one strain each, one isolated from a deer and one from a giraffe.

The genetic relationship between pestiviruses could be made on the basis of the nucleotide sequences of the E2 genes. We compared the 5′-terminal halves of the E2 genes (Table 4). The *Pestivirus* genus could be divided into seven genetic groups. Within the groups the homology was more than 75%. The homology between different genetic groups was 38 to 65% (Table 4). The lowest percentage homology was found between BVDV II and Deer or CSFV. The most closely related genetic groups were the subgroups BVDV Ia and Ib with a percentage homology of 65%. The genetic groups were the same as those found by comparison of the amino acid sequences of the N-terminal halves (see Fig. 2). However, the relationship between antigenic groups was slightly different compared with that of the genetic

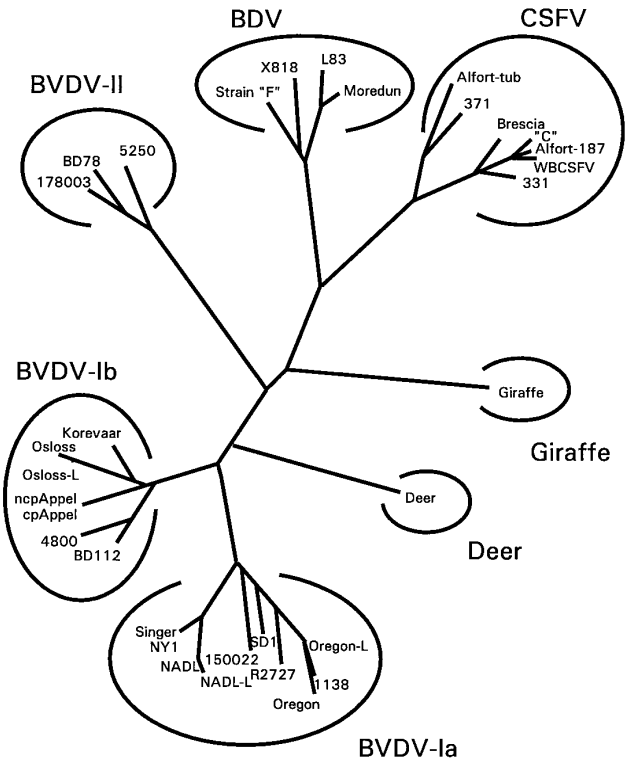

FIG. 2. Subdivision of pestiviruses based on amino acid sequences of the N-terminal half of E2. Numbers correspond to cloned E2 genes of pestiviruses in Table 2. The CSFV group exclusively originates from swine [Brescia, C, 331, 371, WBCSFV, Alfort Tubingen (Alfort-tub), Alfort-187]; the BDV group consists of strains from sheep (L83, Moredun, X818) and a strain from swine (strain F); the BVDV II group consists of viruses isolated from sheep (BD78), swine (5250), and cattle (178003); and the BVDV I group consists of strains originating predominantly from cattle. This BVDV I group is subdivided into subgroup BVDV Ia containing BVDV strains isolated in the United States, such as like NADL or NADL-L (Lelystad) and Oregon or Oregon-L (Lelystad), and some others, such as 150022 from the United Kingdom and 1138 from Germany, and subgroup BVDV Ib containing strain Osloss or Osloss-L (Lelystad) and Dutch strains, such as cpAppel, ncpAppel, BD112, Korevaar, and 4800. Fifth and sixth "groups" consist of one member each: the Deer group with a strain from deer and the Giraffe group with the strain from a giraffe.

groups. Remarkably, the Deer group was now positioned between subgroups BVDV Ia and BVDV Ib, and was more closely related to BVDV Ia (65%) than to BVDV Ib (60%).

DISCUSSION

Using the transient expression system as described (van Rijn *et al.,* 1992, 1993, 1994), the origin of cloned E2 genes could easily be checked with a small panel of MAbs (Tables 2, 3). The levels of detection of transiently expressed $E2^{CSFV}$ protein and viral $E2^{CSFV}$ protein have been shown to be similar (van Rijn *et al.,* 1992). Thus, PCR errors resulting in changes to essential amino acids of epitopes and in amplification of contaminating nucleic acids will be detected by the IPMA result (van Rijn *et al.,* 1994). This was demonstrated in the case of transiently expressed $E2^{NADL-L}$ (pPRKbvd10) (Tables 2, 3). Comparison of the published (NADL) and determined (NADL-L) E2 sequences revealed that pPRKbvd10 encodes an E2 protein with a D → G mutation, which was at the same position as found in an escape variant of strain NADL [D → N on position 838 of NADL (Paton *et al.,* 1992)]. Apparently, the D → G mutation in pPRKbvd10 resulted in the negative immunostaining with MAb 214. Most likely, transiently expressed $E2^{1138}$ contains a mutated epitope for MAb 162 with respect to the viral-encoded E2 protein. Surprisingly, a second independently obtained E2 gene of strain 1138 codes for the same deduced amino acid sequence (data not shown). Possibly, a selected subpopulation of E2 was amplified, or the correct sequence could be found by sequencing more independently obtained cDNAs of $E2^{1138}$. All other transiently expressed E2 proteins showed similar IPMA results as with viral E2, indicating that these E2 genes were correctly amplified (Tables 2 and 3).

Six of seven here defined groups match the neutralization groups defined by Dekker *et al.* (1995). The subdivision is also observed by the study with MAbs, as indeed is the separate nature of the strains from giraffe and deer. Apparently, these phenotypical properties reflect the genetic relationships between viruses. However, based on data presented here, antigenic or neutralization group BVDV I can be divided into subgroups BVDV Ia and BVDV Ib. This subdivision was not clearly observed by the other methods, e.g., cross-neutralization (Dekker *et al.,* 1995) or binding of MAbs (Paton *et al.,* 1995). However, comparison of the nucleotide sequences of the 5′-noncoding region also resulted in a subdivision of BVDV I into BVDV Ia and Ib (Pellerin *et al.,* 1994). Interestingly, this subgrouping seems to be correlated to a geographical separation, although more isolates need to be examined to confirm this. Most strains of BVDV Ia have been isolated in the United States, whereas BVDV Ib consists mainly of strains isolated in continental Europe. This subgrouping, however, is not conclusive. Possibly isolated pestivirus strains, not fitting in the respective subgroup, could be introduced by contaminated bovine serum batches of animals from another part of the world. This assumption is supported by the finding that cell lines of the ATCC collection, human vaccines, and virus stocks have been contaminated with BVDV in the past (Harasawa and Tomiyama, 1994).

Genetic and antigenic relationship is not correlated to host tropism, since groups BVDV I, BVDV II, and BDV contain viruses isolated from different hosts, including ruminants and swine. Furthermore, virulence is also not correlated to this relationship, as the recently isolated high-virulent BVDV type 2 is closely related to the low-virulent strains 5250 and 178003. Possibly, adaptation after transmission to different hosts and escaping from

TABLE 4

Percentages of Similarities and Divergences between Pestivirus Strains Based on the 5′ Halves of Envelope Glycoprotein E2

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	
1		96.3	91.8	92.1	76.0	78.1	80.6	78.4	81.5	79.0	64.7	63.1	64.4	64.2	64.0	62.2	63.1	63.8	47.3	48.2	48.4	43.6	44.1	45.2	43.8	48.3	45.3	46.4	42.9	44.6	42.2	42.8	42.0	1
2	0.9		91.6	92.0	75.2	76.6	78.8	76.6	80.6	78.1	63.8	62.5	63.8	63.6	63.6	61.6	62.5	62.9	46.6	47.7	48.1	43.6	42.8	44.8	44.1	47.5	45.5	46.6	43.1	44.6	42.4	42.9	42.2	2
3	5.6	5.8		96.7	77.9	78.1	80.6	78.4	81.9	79.3	62.7	61.6	64.5	64.4	64.3	61.4	62.7	63.6	46.6	46.2	46.8	43.0	44.4	45.0	45.2	49.2	44.8	45.1	44.0	45.3	43.1	43.9	43.5	3
4	5.3	5.4	0.8		78.6	78.8	81.4	79.3	82.6	80.1	63.4	62.5	64.7	64.5	64.7	61.8	63.1	64.7	47.0	46.6	47.1	42.6	43.9	44.4	44.9	48.6	44.2	44.8	44.2	45.0	42.6	43.3	42.6	4
5	16.1	16.9	14.6	13.8		76.2	78.2	70.2	80.3	78.6	61.9	61.4	64.0	64.0	62.9	57.4	59.5	62.3	43.5	43.9	42.8	38.1	38.6	38.8	37.7	45.6	43.2	43.3	43.3	41.8	41.3	41.7	40.7	5
6	15.8	16.5	15.0	14.3	15.4		95.1	93.1	83.7	84.6	61.4	59.6	61.4	61.2	61.6	59.4	60.3	63.8	44.7	43.4	44.2	38.4	39.3	41.0	39.9	47.2	45.0	42.9	44.4	43.7	42.2	43.1	41.8	6
7	15.4	16.3	14.6	13.9	15.0	0.4		95.4	86.1	87.0	63.8	62.0	63.8	63.6	63.6	60.5	62.7	65.6	45.1	43.4	43.8	39.3	40.0	41.5	40.4	47.7	45.1	42.8	44.2	43.5	42.0	42.9	41.7	7
8	17.3	18.2	16.5	15.8	15.9	2.4	2.1		84.3	85.0	63.8	61.6	63.3	63.3	63.4	59.6	61.8	65.1	44.0	42.7	37.3	38.8	39.7	41.3	39.3	47.2	43.5	41.5	43.3	41.8	42.0	43.1	41.8	8
9	14.6	15.2	14.3	13.5	13.6	11.1	10.7	12.6		84.6	66.4	64.2	66.4	66.2	65.4	63.3	64.9	67.3	43.8	40.1	39.0	41.5	41.5	42.2	41.7	48.3	46.1	45.0	43.3	44.2	43.3	44.2	43.3	9
10	16.7	17.3	15.9	15.2	14.6	10.5	10.1	12.0	11.8		64.0	61.1	63.3	63.1	64.2	61.4	63.1	64.0	44.4	39.6	44.0	42.8	44.6	45.2	43.0	47.0	47.0	44.6	45.3	44.2	43.1	44.0	42.9	10
11	29.7	30.5	30.3	29.7	28.5	29.2	28.8	28.9	26.9	29.1		88.8	78.8	78.6	77.6	76.4	79.2	59.6	41.6	40.9	41.4	41.5	44.6	43.5	41.5	44.2	42.2	42.8	41.1	40.2	40.0	40.7	39.6	11
12	30.8	31.2	30.8	30.3	28.8	31.1	30.6	31.0	28.8	31.2	8.3		78.4	78.2	78.7	75.9	78.4	58.7	42.7	42.1	43.4	41.5	44.1	43.1	40.4	43.5	40.0	43.1	41.5	39.4	39.8	39.4	40.2	12
13	29.8	30.1	29.0	28.8	26.4	29.1	28.6	29.2	27.1	28.8	16.7	17.5		97.3	78.7	78.8	81.4	60.1	43.4	39.2	43.6	45.6	45.2	44.8	44.5	47.3	43.1	43.7	39.1	39.3	39.6	40.0	38.7	13
14	29.9	30.3	29.2	29.0	26.4	29.2	28.8	29.2	27.3	29.0	16.9	17.7	0.2		78.7	78.6	81.2	60.0	43.4	39.2	43.6	45.4	45.0	44.8	44.3	47.5	42.9	43.5	39.1	39.3	39.6	40.0	38.7	14
15	28.9	29.3	29.1	28.7	26.9	28.8	28.4	28.5	28.0	28.0	16.1	15.5	14.5	14.5		79.2	81.6	59.6	42.9	44.5	46.6	44.7	43.4	43.8	44.9	47.1	45.0	47.1	41.4	40.8	40.3	39.7	40.1	15
16	29.9	30.3	30.9	30.5	30.7	30.8	30.3	31.1	29.2	29.8	16.2	17.5	15.3	15.4	13.4		94.3	55.8	42.5	43.1	42.1	42.6	44.6	44.1	42.6	45.2	40.6	42.4	38.3	37.8	38.2	37.8	37.4	16
17	30.3	30.7	31.5	31.1	30.3	30.6	30.1	30.9	29.4	29.9	16.4	17.5	14.8	15.0	12.8	0.4		57.8	44.5	44.5	43.4	43.4	45.3	44.8	43.6	46.8	41.3	42.8	38.3	38.0	38.7	38.3	37.8	17
18	27.4	28.0	28.0	27.3	26.3	27.7	27.4	27.4	25.8	27.6	30.9	32.4	31.7	31.9	31.3	32.8	32.5		42.1	42.5	41.2	39.2	39.7	41.0	40.3	46.4	41.1	41.8	41.5	39.8	39.6	40.2	38.9	18
19	40.1	40.5	40.5	40.3	40.2	41.3	41.1	41.5	42.3	41.5	42.9	43.5	42.2	42.2	42.2	42.6	42.4	43.1		89.6	85.0	39.0	37.0	38.4	37.9	38.6	38.3	37.3	36.6	39.4	36.2	36.6	35.5	19
20	38.5	38.9	39.1	38.9	39.8	41.1	40.9	41.1	42.5	41.1	42.5	42.7	41.6	41.6	40.8	41.5	41.0	42.1	6.5		87.2	41.4	39.7	39.9	39.0	39.6	38.4	37.0	36.4	39.0	36.0	35.9	34.9	20
21	38.9	38.9	39.7	39.5	41.6	40.9	40.9	41.5	43.5	40.7	43.1	42.9	42.2	42.2	40.2	42.0	41.6	44.1	11.6	8.7		42.0	38.6	40.7	38.4	38.8	38.8	38.3	38.6	39.7	37.5	37.9	37.5	21
22	44.8	45.0	45.0	45.6	46.0	47.6	47.6	47.9	45.8	44.2	46.2	46.2	43.6	43.8	45.4	45.9	45.7	47.3	46.0	44.2	44.6		77.2	76.5	79.6	43.9	54.4	56.1	52.9	54.6	52.9	53.3	53.5	22
23	44.1	44.9	44.3	44.5	45.5	47.5	47.2	47.6	45.5	42.9	45.3	45.6	44.6	44.8	46.2	44.8	44.8	46.5	47.2	46.0	45.8	15.2		92.0	78.7	47.9	49.7	52.8	50.6	51.2	47.5	48.3	49.5	23
24	43.5	43.9	43.7	43.9	44.9	45.9	46.0	46.0	45.1	43.1	45.3	46.0	44.0	44.0	45.8	45.2	45.2	46.3	47.0	45.6	45.0	15.6	5.3		76.5	48.1	51.0	53.0	50.1	51.2	48.6	49.0	50.3	24
25	45.1	45.3	44.5	44.7	46.0	47.4	47.3	47.9	45.9	44.3	47.3	47.9	45.2	45.4	45.9	46.0	45.8	47.0	48.5	46.9	46.9	15.5	14.2	15.4		46.9	55.1	57.0	54.6	55.7	52.2	52.0	52.9	25
26	39.8	40.3	39.2	39.4	39.4	40.0	39.6	40.2	39.4	40.2	42.9	44.1	41.8	41.6	40.4	41.4	41.2	40.2	47.3	46.6	46.7	46.2	45.1	44.7	44.3		40.2	43.7	41.7	41.1	40.7	40.6	41.5	26
27	43.0	42.8	44.3	44.7	42.4	44.6	44.7	45.5	43.4	42.4	45.5	46.5	44.4	44.6	43.8	46.0	45.4	46.0	48.1	47.5	47.7	36.1	39.2	37.5	35.9	47.8		85.3	78.5	80.2	81.1	80.7	81.7	27
28	43.2	43.0	44.7	45.1	43.6	46.2	46.3	46.9	44.9	44.7	45.7	45.7	45.4	45.6	43.0	45.6	44.8	46.0	49.5	48.3	48.5	35.7	37.7	36.5	35.3	45.7	11.1		79.6	81.8	80.0	79.6	80.9	28
29	45.1	44.9	44.5	44.7	42.0	44.4	44.3	44.3	44.5	43.0	45.5	45.5	46.4	46.4	46.7	47.0	47.0	45.4	49.5	48.7	47.7	37.8	38.7	37.9	37.4	47.6	18.1	17.5		88.8	87.9	88.3	88.8	29
30	43.4	43.2	43.2	43.6	42.6	44.6	44.7	45.3	43.4	43.8	46.1	46.7	46.4	46.4	46.5	47.4	47.0	46.6	46.9	46.3	45.7	37.3	39.4	38.5	36.6	47.3	16.6	15.1	8.5		88.6	88.3	89.4	30
31	44.9	44.7	45.1	45.5	44.0	45.8	45.9	45.9	44.5	44.7	46.9	46.9	46.6	46.6	47.3	48.0	47.6	47.4	49.1	48.5	47.9	38.0	41.0	39.6	38.3	48.2	15.3	16.4	9.6	8.3		94.3	93.9	31
32	44.4	44.2	44.4	44.8	43.1	45.3	45.4	44.6	44.0	44.2	46.0	47.2	45.7	45.7	47.6	47.7	47.3	46.9	48.6	47.8	47.2	37.5	39.8	38.7	38.5	47.9	15.2	16.7	8.7	8.2	2.7		94.5	32
33	44.7	44.5	45.1	45.5	44.0	46.0	46.1	45.7	44.7	44.9	46.7	46.7	47.2	47.2	47.5	47.8	47.4	47.7	49.3	48.3	47.1	37.3	39.4	38.3	37.8	47.3	14.7	15.8	8.7	7.7	3.6	2.5		33
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	

01.SEQ 02.SEQ 03.SEQ 04.SEQ 05.SEQ 06.SEQ 07.SEQ 08.SEQ 09.SEQ 10.SEQ 11.SEQ 12.SEQ 13.SEQ 14.SEQ 15.SEQ 16.SEQ 17.SEQ 18.SEQ 19.SEQ 20.SEQ 21.SEQ 22.SEQ 23.SEQ 24.SEQ 25.SEQ 26.SEQ 27.SEQ 28.SEQ 29.SEQ 30.SEQ 31.SEQ 32.SEQ 33.SEQ

Note. Group BVDV-Ia: (1) NADL, (2) NADL-L, (3) NY1, (4) Singer, (5) 150022, (6) Oregon, (7) Oregon-L (8) 1138, (9) SD1, (10) R2727. Group BVDV-Ib; (11) BD112, (12) 4800, (13) cpAppel, (14) ncpAppel, (15) Korevaar, (16) Osloss, (17) Osloss-L. Group Deer: (18) Deer. Group BVDV-II: (19) 178003, (20) BD78, (21) 5250. Group BDV: (22) F, (23) L83, (24) Moredun, (25) X818. Group Giraffe: (26) Giraffe. Group CSFV: (27) Alfort-tub, (28) 371, (29) Brescia, (30) 331, (31) C, (32) Alfort-187, (33) WBCSFV.

different immune systems have enlarged the diversity of these pestiviruses.

CSFV is readily distinguishable from other pestiviruses. First, CSFV is restricted to swine, whereas other pestivirus strains have been associated with field infections of different ruminants as well as swine. Second, $E2^{CSFV}$ contains conserved and CSFV-specific epitopes (Edwards *et al.,* 1991; Wensvoort 1989b), whereas E2 proteins of other pestiviruses are a diverse group and do not contain conserved epitopes. Furthermore, the groups of pestiviruses defined here do not contain conserved and specific epitopes on E2, although it is not excluded that such epitopes exist. Third, CSFV strains can be differentiated from other pestiviruses by reverse transcription PCR, which was based on protein-encoding regions of the pestivirus genome (Katz *et al.,* 1993; Lowings *et al.,* 1994; Hooft van Iddekinge *et al.,* 1995). Fourth, pestiviruses contain two extra cysteines in the immunogenic N-terminal half of E2 with respect to CSFV strains (Fig. 1). Possibly these two cysteines drastically affect the overall structure of E2. However, since some amino acid changes in escape variants of CSFV and BVDV are located on similar positions, a similar overall structure of $E2^{CSFV}$ and $E2^{BVDV}$ has been suggested (van Rijn *et al.,* 1994).

Previous reports have shown a division of the *Pestivirus* genus into four groups: CSFV, BVDV I (classical BVDV), BVDV II (atypical BVDV), and BDV (Becher *et al.,* 1995; Paton *et al.,* 1995; Sullivan *et al.,* 1997; Tijssen *et al.,* 1996; Wensvoort *et al.,* 1989b). Since this grouping was also based on genetic data, these groups have been accepted as genotypes of the *Pestivirus* genus. On the basis of binding of MAbs, the strains from deer and giraffe were most closely related to BVDV, although they were unreactive with the majority of MAbs raised against BVDV. Preliminary data showed strain Deer to be intermediate between BVDV Ia and BVDV Ib and strain Giraffe to be quite distinct from BVDV I (Paton *et al.,* 1995). We have shown that the homology between strain Giraffe and the other pestiviruses is less than 50%. This indicates that the Giraffe group is a newly discovered genotype within the *Pestivirus* genus, which can be designated pestivirus 5. By cross-neutralization assays, Dekker *et al.* (1995) have concluded that, besides strain Giraffe, strain Deer is a distinct neutralization "group" of the *Pestivirus* genus. Based on the genetic relationship, the deer strain is as distinct from BVDV Ia as BVDV Ib is from BVDV Ia. However, by comparison of the amino acid sequences of E2, the percentage homology of the deer strain and BVDV Ia is intermediate between that of BVDV Ia and BVDV Ib and that of defined genotypes. Findings of Paton *et al.* (1995) and Dekker *et al.* (1995) support the proposal to define the Deer group as a new genotype of the *Pestivirus* genus and designate it pestivirus 6. Further, BVDV I can be divided into two subgroups, BVDV Ia and BVDV Ib, which is supported by the data of Pellerin *et al.* (1994). These subgroups demonstrate a genetic homology of 65%, which is comparable to that between Deer and BVDV I. However, on the level of the amino acid sequence the homology between BVDV Ia and BVDV Ib is higher than between BVDV I and Deer. So far, BVDV Ia and BVDV Ib cannot be distinguished by cross-neutralization experiments and MAb binding. Taken together, we propose to divide genotype BVDV I into subtypes BVDV Ia and BVDV Ib, as this has been proposed for the genotype CSFV by Lowings *et al.* (1996).

In this report, for the first time, E2 genes encompassing pestivirus strains of all known neutralization groups and from different hosts were studied. The investigated pestiviruses were isolated from swine and several ruminants including cattle, sheep, deer, and giraffe. By comparison of the E2 gene, the most variable and immunodominant protein of pestiviruses, six genotypes of the *Pestivirus* genus are found. Further, the genotype BVDV I can be divided into two subtypes, BVDV Ia and BVDV Ib.

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REFERENCES

Barlow, R. M. (1972). Experiments in border disease. IV. Pathological changes in ewes. *J. Comp. Pathol.* 82, 151–157.

Becher, P., Shannon, A. D., Tautz, N., and Thiel, H.-J. (1994). Molecular characterization of border disease virus, a pestivirus from sheep. *Virology* 198, 542–551.

Becher, P., Konig, M., Paton, D. J., and Thiel, H.-J. (1995). Further characterization of border disease virus isolates: Evidence for the presence of more than three species within the genus pestivirus. *Virology* 209, 200–206.

Bolin, S. R., Moennig, V., Kelso-Gourley, N. E., and Ridpath, J. F. (1988). Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. *Arch. Virol.* 99, 117–123.

Bruschke, C. J. M., van Rijn, P. A., Moormann, R. J. M., and van Oirschot, J. T. (1996). Antigenically different pestivirus strains induce congenital infection in sheep: a model for bovine virus diarrhea virus vaccine efficacy studies. *Vet. Microbiol.* 50, 33–43.

Caij, A., Muyldermans, G., De Smet, A., Hamers, R., and Koenen, F. (1993). Production and characterization of monoclonal antibodies against hog cholera virus (Alfort-187 Strain). *Arch. Virol.* 131, 185–192.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.

Collett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K., and A. F. Purchio. (1988). Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology* 165, 191–199.

Dekker, A., Wensvoort, G., and Terpstra, C. (1995). Six antigenic groups within the genus pestivirus as identified by cross neutralization assays. *Vet. Microbiol.* 47, 317–329.

Deng, R., and Brock, K. V. (1992). Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. *Virology* 191, 867–879.

Edwards, S., Sands, S. S., and Harkness, J. W. (1988). The aplication of monoclonal antibody panels to characterize pestivirus isolates from ruminants in Great Britain. *Arch. Virol.* 102, 197–206.

Edwards, S., Moennig, V., and Wensvoort, G. (1991). The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. *Vet. Microbiol.* 29, 101–108.

Hanahan, D. (1985). in DNA Cloning. A Practical Approach, Techniques for transformation of *E. coli* I. Glover, D. M. ed. (IRL press), Oxford 109–135.

Harasawa, R. (1994). Comparative Analysis of the 5′ Non-Coding Region of Pestivirus RNA Detected from Live Virus Vaccines. *J. Vet. Med. Sc.* 56, 961–964.

Harasawa, R., and Tomiyama, T. (1994). Evidence of pestivirus RNA in human vaccines. *J. Clin. Microbiol.* 32, 1604–1605.

Harpin, S., Elahi, S. M., Cornaglia, E., Yolken, R. H., and Elazhary, Y. (1995). The 5′-untranslated region sequence of a potential new genotype of bovine viral diarrhea virus. *Arch. Virol.* 140, 1285–1290.

Hofmann, M. A., Brechtbühl, K., and Stauber, N. (1994). Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5′-noncoding region. *Arch. Virol.* 139, 217–229.

Hooft van Iddekinge, B. J. L., van Rijn, P. A., and Moormann, R. J. M. (1995). Application of the Polymerase Chain Reaction to the Detection of Bovine Viral Diarrhea Virus Infection in Cattle. PCR: Protocols for Diagnosis of Human and Animal Virus Diseases. Y. Becker and G. Darai (eds). Springer Verlag Berlin-Heidelberg. 463–472.

Hulst, M. M., Westra, D. F., Wensvoort, G., and Moormann, R. J. M. (1993). Glycoprotein E1 of Hog Cholera Virus expressed in Insect Cells protects Swine from Hog Cholera. *J. Virol.* 67, 5435–5442.

Ishikawa, K., Nagai, H., Katayama, K., Tsutsui, M., Tanabayashi, K., Takeuchi, K., Hishiyama, M., Saitoh, A., Takagi, M., Gotoh, K., Muramatsu, M., Yamada, A. (1995). Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE- and the wild-type parental strain ALD. *Arch. Virol.* 140, 1385–1391.

Katz, J. B., Ridpath, J. F., and Bolin, S. R. (1993). Presumptive diagnostic differentiation of hog cholera virus from bovine viral diarrhea and border disease viruses by using a cDNA nested-amplification approach. *J. Clin. Microbiol.* 31, 565–568.

König, M., Lengsfeld, T., Pauly, T., Stark, R., and Thiel, H.-J. (1995). Classical Swine Fever Virus: Independent Induction of Protective Immunity by Two Structural Glycoproteins. *J. Virol.* 69, 6479–6486.

Lowings, J. P., Paton, D. J., Sands, J. J., De Mia, G. M., and Rutili, D. (1994). Classical swine fever: genetic detection and analysis of differences between virus isolates. *J. Gen. Virol.* 77, 1311–1321.

Lowings, J. P., Ibata, G., Needham, J., and Paton, D. J. (1996). Classical swine fever virus diversity and evolution. *J. Gen. Virol.* 77, 1131–1321.

Meyers, G., Rümenapf, T., and Thiel, H.-J. (1989). Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology* 171, 555–567.

Moennig, V., and Plagemann, P. G. W. (1992). The Pestiviruses. *Advances in Virus Research* 41, 53–98.

Moennig, V., Bolin, S. R., Coulibaly, C. O. Z., Kelso Gourley, N. E., Liess, B., Mateo, A., Peters, W., and Greiser-Wilke, I. (1987). Studies into the antigenic structure of pestiviruses using monoclonal antibodies. *Deutsche Tierärztliche Wochenschrift* 94, 572–576.

Moormann, R. J. M., Warmerdam, P. A., van der Meer, B., Schaaper, W. M. M., Wensvoort, G., and Hulst, M. M. (1990). Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. *Virology* 177, 184–198.

Moormann, R. J. M., van Gennip, H. G. P., Miedema, G. K. W., Hulst, M. M., and van Rijn, P. A. (1996). Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. *J. Virol.* 70, 763–770.

Paton, D. J., Sands, J. J., and Roehe, P. M. (1991). BVD monoclonal antibodies: Relationship between viral protein specificity and viral strain specificity. *Arch. Virol.* (Suppl. 3), 47–54.

Paton, D. J., Lowings, J. P., and Barrett, A. D. T. (1992). Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. *Virology* 190, 763–772.

Paton, D. J., Sands, J. J., and Edwards, S. (1994). Border disease: delineation by monoclonal antibodies. *Arch. Virol.* 135, 241–252.

Paton, D. J., Lowings, J. P., Sands, J. J., Smith, J. E., Ibata, G., and Edwards, S. (1995). A Proposed Division of the Pestivirus Genus Using Monoclonal-Antibodies, Supported by Cross-Neutralization Assays and Genetic Sequencing. *Vet. Res.* 26, 92–109.

Pellerin, C., Van den Hurk, J., Lecomte, J., and Tijssen, P. (1994). Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. *Virology* 203, 260–268.

Peters, W., Greiser-Wilke, I., Moennig, V., and Liess, B. (1986). Preliminary serological characterization of bovine viral diarrhoea virus strains using monoclonal antibodies. *Vet. Microbiol.* 12, 195–200.

Plowright, W. (1969). Other virus diseases in relation to the JP15 programme. In: Joint Campaign Against Rinderpest. Proceedings of the 1st technical review meeting, phase IV, Mogadiscio. Organization of African Unity, Kenya, 19–23.

Poole, T. L., Wang, C., Popp, R. A., Potgieter, L. D. N., Siddiqui, A., and Collett, M. S. (1995). Pestivirus Translation Initiation Occurs by Internal Ribosome Entry. *Virology* 206, 750–754.

Renard, A., Dina, D., and Martial, J. A. (1987). Complete nucleotide sequence of bovine viral diarrhoea virus genome and its fragment, useful for making antigenic proteins in therapy and diagnosis. European Patent Application, no. 0208672.

Ridpath, J. F., Bolin, S. R., and Dubovi, E. J. (1994). Segregation of Bovine Viral Diarrhea Virus into Genotypes. *Virology* 205, 66–74.

Rijnbrand, R., van der Straaten, T., van Rijn, P. A., Spaan, W. J. M., and Bredenbeek, P. J. (1997). The 5′ noncoding region of classical swine fever virus directs internal entry of ribosomes. *J. Virol.* 71, 451–457.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, Cold Spring Harbor, NY.

Sullivan, D. G., Chang, G.-J., Trent, D. W., and Akkina, R. K. (1994). Nucleotide sequence analysis of the structural coding region of the pestivirus border disease virus. *Virus Res.* 33, 219–228.

Sullivan, D. G., Chang, G.-J., and Akkina, R. K. (1997). Genetic characterization of ruminant pestiviruses: sequence analysis of viral genotypes isolated from sheep. *Virus Res.* 47, 19–29.

Terpstra, C. (1989). Swine kidney cell culture and its use in vaccine production. European patent application 89201574.4. Publication 0 351 901 B1.

Tijssen, P., Pellerin, C., Lecomte, J., and Van den Hurk, J. (1996). Immunodominant E2 (gp53) sequences of highly virulent bovine viral diarrhea group II viruses indicate a close resemblance to a subgroup of border disease viruses. *Virology* 217, 356–361.

Van Rijn, P. A., van Gennip, H. G. P., de Meijer, E. J., and Moormann, R. J. M. (1992). A preliminary map of epitopes on envelope glycoprotein E1 of HCV strain Brescia. *Vet. Microbiol.* 33, 221–230.

Van Rijn, P. A., de Meijer, E. J., van Gennip, H. G. P., and Moormann, R. J. M. (1993). Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. *J. Gen. Virol.* 74, 2053–2060.

Van Rijn, P. A., Miedema, G. K. W., Wensvoort, G., van Gennip, H. G. P., and Moormann, R. J. M. (1994). Antigenic structure of Envelope Glycoprotein E1 of Hog Cholera virus. *J. Virol.* 68, 3934–3942.

Van Rijn, P. A., Bossers, A., Wensvoort, G., and Moormann, R. J. M. (1996). Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. *J. Gen. Virol.* 77, 2737–2745.

Van Zijl, M., Wensvoort, G., de Kluyver, E. P., Hulst, M. M., van der Gulden, H., Gielkens, A. L. J., Berns, A., and Moormann, R. J. M. (1991). Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. *J. Virol.* 65, 2761–2765.

Vilcek, S., Herring, A. J., Nettleton, P. F., Lowings, J. P. L., and Paton, D. J. (1994). Pestiviruses isolated from pigs, cattle and sheep can be

allocated into three genotypes using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.* 136, 309–323.

Weiland, E., Stark, R., Haas, B., Rümenapf, T., and Thiel, H.-J. (1990). Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. *J. Virol.* 64, 3563–3569.

Weiland, E., Ahl, R., Stark, R., Weiland, F., and Thiel, H.-J. (1992). A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. *J. Virol.* 66, 3677–3682.

Wensvoort, G. (1989a). Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. *Journal of General Virology* 70, 2685–2876.

Wensvoort, G. (1989b). Epitopes on structural proteins of hog cholera (swine fever) virus. PhD thesis, State University of Utrecht, Utrecht, The Netherlands.

Wensvoort, G., Terpstra, C., Boonstra, J., Bloemraad, M., and van Zaane, D. (1986). Production of monoclonal antibodies against swine fever and their use in laboratory diagnosis. *Vet. Microbiol.* 12, 101–108.

Wensvoort, G., Terpstra, C., de Kluijver, E. P., Kragten, C., and Warnaar, J. C. (1989a). Antigenic differentiation of pestivirus strains with monoclonal antibodies against hog cholera virus. *Vet. Microbiol.* 21, 9–20.

Wensvoort, G., Terpstra, C., and Kluyver, E. P. (1989b). Characterization of Porcine and Some Ruminant Pestiviruses by Cross-neutralization. *Vet. Microbiol.* 20, 291–306.

Wirz, B., Tratschin, J., Muller, H. K., and Mitchell, D. B. (1993). Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reactions. *J. of Clin. Microbiol.* 31, 1148–1153.